Purification and Properties of Lipid A Disaccharide Synthase of Escherichia coli*

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Lipid A disaccharide synthase of Escherichia coli catalyzes the reaction 2,3-diacyl-GlcN-1-P + UDP-2',3'-diacyl-GlcN \rightarrow 2',3',2',3'-diacyl-GlcN(6,1') \rightarrow 6(2,3-diacyl-GlcN-1-P + UDP (Ray, B. L., Painter, G., and Raet, C. R. H. (1984) J. Biol. Chem. 259, 4852–4859). Using a strain that overproduces the enzyme about 200-fold we have devised a simple purification to near homogeneity, utilizing two types of dye-ligand resins and heparin-agarose. The overall purification starting with membrane-free extracts was 54-fold (16,000-fold relative to wild-type extracts) with a 31% yield. The subunit molecular mass determined by sodium dodecyl sulfate gel electrophoresis is approximately 42,000 daltons, and the native enzyme appears to be a dimer. The amino-terminal sequence is Met-Thr-Glu-Gln-Arg-Pro-Leu-Thr-Ile-Ala... consistent with the results predicted from the DNA sequence, Met-Thr-Glu-Gln-Arg-Pro-Leu-Thr-Ile-Ala...

The purified enzyme displays a strong kinetic preference for sugar substrates bearing two fatty acyl moieties, but it is, nevertheless, very useful for the semisynthetic preparation of many lipid A analogs. Gel filtration studies demonstrate that the natural substrates (2,3-diacyl-GlcN-1-P and UDP-2',3'-diacyl-GlcN) form micelles ($n \approx 300$), rather than bilayers, under conditions used to assay the enzyme. Unlike most enzymes of glycerophospholipid biosynthesis, the lipid A disaccharide synthase does not require the presence of a detergent for catalytic activity. At 1 mM UDP-2,3-diacyl-GlcN the $V_{max}$ and $K_m$ values for 2,3-diacyl-GlcN-1-P are $14,028 \pm 513$ nmol/min/mg and 0.27 ± 0.02 mm. When 2,3-diacyl-GlcN-1-P is maintained at 1 mM, they are $12,368 \pm 472$ nmol/min/mg and 0.11 ± 0.01 mm for UDP-2',3'-diacyl-GlcN.

Recent studies in our laboratory have demonstrated the existence of a previously unrecognized class of phospholipids in Escherichia coli containing glucosamine instead of glycerol as their backbone (1–4). These substrates define a novel biosynthetic pathway (Scheme 1), the end product of which is the lipid A component of lipopolysaccharide (1–3). The lipid A moiety accounts for about 30% of the fatty acyl moieties present in the E. coli envelope (3) and makes up the outer monolayer of the outer membrane of E. coli and related Gram-negative bacteria (3, 5).

Although the covalent structures of all the early intermediates (Scheme 1) have been thoroughly established (1–4, 6–11), none of the enzymes involved in their interconversions has been characterized. With the exception of the 4' kinase (10), they are all recovered in the cytosolic fraction (7, 8, 11). In contrast, most enzymes involved in the biosynthesis of the classical glycerophospholipids are integral membrane proteins (3).

We now report the purification to near homogeneity of the E. coli lipid A disaccharide synthase (7), a protein encoded by the $pxB$ gene (12, 13). Previous molecular cloning has made it possible to overproduce the synthase several hundred-fold (13), and the DNA sequence of the $pxB$ gene is known (14). The purified enzyme is a useful reagent for the preparation of lipid A substructures and analogs (15).

EXPERIMENTAL PROCEDURES

Growth of Bacteria Used to Isolate the Disaccharide Synthase—E. coli MC1061/pSR8, an overproducer of lipid A disaccharide synthase, was previously isolated by Crowell et al. (13). One liter of cells in LB broth (16) containing 50 µg/ml ampicillin was grown overnight at 30 °C using a rotary shaker for aeration. This inoculum was used to start the growth of a 30-liter LB broth culture supplemented with 70 g of K$_2$HPO$_4$, 30 g of KH$_2$PO$_4$, 300 g of fructose, and 1.5 g of ampicillin. After 2.5 h of growth at 30 °C with aeration in a New Brunswick fermentor, the A$_{600}$ was 1.0. Next, 150 g of L-arabinose were added, and the induction of the enzyme was achieved by growing the bacteria for 8 more hours under the same conditions. The cells were harvested by centrifugation, and the paste ($166$ g) was stored at −80 °C.

Purification of Lipid A Disaccharide Synthase—Four grams of E. coli MC1061/pSR8 were suspended in 20 ml of 0.01 M potassium phosphate, pH 7.0, and broken in a cold French pressure cell at 18,000 p.s.i. All the following operations were conducted at 4 °C. The broken cells were removed by centrifugation at 10,000 × g for 15 min, and the membrane-free supernatant was prepared by centrifugation at 100,000 × g for 2 h. The supernatant was adjusted to a volume of 45 ml so that it contained 0.01 M potassium phosphate, pH 7.0, 0.2 M KCl, 0.05% Triton X-100, and 20% glycerol.

A 60-ml column (2.5-cm diameter) of Matrex Gel Blue B (Amicon Corp.) derivatized with 0.080 mg of ligand/ml of gel was equilibrated by washing with 20 column volumes of 0.01 M potassium phosphate, pH 7.0, containing 20% glycerol, 0.05% Triton X-100, and 0.2 M KCl at a flow rate of 1.4 ml/min. The supernatant, described above, was applied to the column at the same flow rate, and the column was washed with the same buffer. A total of six fractions (30 ml each) were collected. The enzyme was eluted with a buffer identical to the application buffer, except that it contained 2.5 M KCl. Eight more 30-ml fractions were collected.

A 30-ml column (2.5-cm diameter) of Reactive Red 120-agarose (Sigma) derivatized with 0.96 µmol of ligand/ml of gel was equilibrated by washing with 20 column volumes of the same application buffer as above. Fractions 9–13 (containing the disaccharide synthase) from the Matrex Gel Blue B column were pooled and diluted to 1875 ml, in order to adjust the salt concentration to 0.2 M KCl, while keeping the concentration of all other components the same as...
The lpxB Gene Product

**SCHEME 1. Pathway for the biosynthesis of lipid A in E. coli.**
The product of the lpxB gene catalyzes the condensation of one molecule of UDP-2,3-diacylglyceramine with one molecule of lipid X to generate the 2-1-6 linkage that is characteristic of lipid A. Evidence for this scheme has been presented previously (1-3, 6-14). ACP, acyl carrier protein; KDO, 3-deoxy-d-manno-octulosonate.

in the application buffer. Next, the sample was applied to the Reactive Red column at 1.8 ml/min, and then the column was washed with 75 ml of the application buffer. The enzyme was eluted with a buffer identical to the application buffer except containing 2.5 M KCl. Eight fractions (12.5 ml each) were collected. Fractions 4-8 from the Reactive Red 120-agarose were pooled and diluted to 782 ml to make the salt concentration 0.2 M KCl, while keeping the concentration of all other components the same as in the application buffer. The sample was applied to this column at 1.8 ml/min, and the column was washed with 60 ml of the application buffer. The enzyme was eluted with a buffer identical to the application buffer except containing 2.5 M KCl. Six fractions of 10 ml each were collected. The purified enzyme samples were frozen in liquid nitrogen and stored at -80 °C.

**Assay of Lipid A Disaccharide Synthase**—A qualitative assay of enzyme activity was performed as follows. Reactions were conducted in a total final volume of 25 μl containing 1 mM UDP-2,3-diacyl-GlcN, \(1 \text{ mm 2,3-diacyl-GlcN-1-P (lipid X), and 10 mM Hepes, adjusted to pH 8.0 with KOH. (Lipid substrates were dispersed together at 2 mM each in 10 mM Hepes, pH 8, with the aid of a bath sonicator (Laboratory Supplies Co., Inc., Hicksville, NY) for 2 min at 23 °C before dilution into the assay.) Assays were usually started by the addition of 10 μl of enzyme, suitably diluted in 1 mg/ml fatty acid-free bovine serum albumin in 10 mM Hepes, pH 8.0. Blanks were started by the addition of 10 μl of bovine serum albumin (1 mg/ml) in 10 mM Hepes, pH 8. The reaction mixture was incubated at 37 °C for 20 min, at the end of which time 5 μl was spotted on a 5 × 10-cm silica gel F (250 μm) thin layer chromatography plate (E. Merck, Darmstadt, Federal Republic of Germany). After drying the spots under a cold air stream, the plate was developed in the solvent chloroform:methanol:water:acetic acid (25:15:4:2, v/v), and then it was sprayed with 10% H2SO4 in ethanol and charred on a hot plate to visualize the two substrates and the more rapidly migrating product (7). The intensities of charring provided a reliable qualitative measure of enzyme activity.

The quantitative assay of the enzyme activity involved the use of \(^{32}\text{P}\)-labeled lipid X (see below). Each assay tube contained approximately 25,000 cpm of \(^{32}\text{P}\)-lipid X (10 cpm/nmol). Assay conditions were the same as for the qualitative assay. The reaction was stopped by pipetting 20 μl of the reaction mixture into a two-phase neutral Bligh-Dyer system (12) (chloroform, 2 ml; methanol, 2 ml; and phosphate-buffered saline, 1.8 ml). After mixing in a screw-cap glass tube equipped with a Teflon-lined cap, the lower phase of the two-phase system was separated by centrifugation for 5 min in a clinical centrifuge and washed twice with 5.8 ml of a fresh, pre-equilibrated, neutral Bligh-Dyer upper phase. One ml of the lower phase was transferred with a glass pipette into a scintillation vial. The chloroform was dried in a stream of hot air, 10 ml of the scintillant Bio Safe II (RPI, Mount Pleasant, Ill) was added, and the sample was counted in a Packard Tri-Carb 3255 scintillation counter.

For kinetic experiments, the concentrations of lipid X, UDP-2,3-diacylglyceramine, and Triton X-100 were varied, as described under "Results," and in all cases, substrates were mixed and subjected to a 2-min sonic irradiation (as above) prior to the addition of enzyme. (Sonic irradiation did not greatly influence the rate of the reaction, but it did slightly improve the reproducibility of the assay, and it prevented the lipids from binding to the walls of the reaction vessel.)

Preparation of \(^{32}\text{P}\)-Lipid X—A 10-ml culture of strain M77 (17) in G56 medium (18) was started with a 50-μl aliquot of an overnight culture grown at 30 °C. The culture was incubated on a rotary shaker at 30 °C until the \(A_{550}\) reached 0.3. The cells were then pelleted in a clinical centrifuge at the highest setting for 15 min. The cell pellet was resuspended in 10 ml of fresh G56 medium without phosphate, and \(^{32}\text{P}\) was added at 100 μCi/ml. The culture was then transferred to a 42 °C rotary shaker bath for 3 h. The radiolabeled cells were pelleted in the clinical centrifuge and resuspended in 0.8 ml of phosphate-buffered saline (10), pH 7.3. After transferring to a glass tube, 1 ml of CHCl3 and 2 ml of methanol were added. The resulting cell debris was collected by centrifugation and discarded. To the supernatant, 1 ml of CHCl3 and 1 ml of phosphate-buffered saline were added, forming a two-phase Bligh and Dyer system at neutral pH. The majority of the \(^{32}\text{P}\)-lipid X partitioned into the upper phase under these conditions. The upper phase was washed three times with phosphate-buffered saline-pre-equilibrated lower phase, and the lower phases were discarded. The \(^{32}\text{P}\)-lipid X was then partitioned into the lower phase by the addition of the upper phase of 50 μl of concentrated HCl and 2 ml of acid pre-equilibrated lower phase. The lower phase was then washed twice with acid pre-equilibrated upper phase. The washed lower phase was evaporated under a stream of \(N_2\) and the residue was taken up in 2 ml of CHCl3/methanol (2:1, v/v). The \(^{32}\text{P}\)-lipid X solution was stored at -80 °C. The final yield of this preparation was 2 × 10⁶ cpm of \(^{32}\text{P}\)-lipid X/mg of protein.

1 The abbreviations and trivial names used are: UDP-2,3-diacyl-GlcN, UDP, N,O-bis[(R)-3-hydroxytetradecanoyl]-o-d-glucosamine; UDP-3-O-(3-hydroxymyristoyl)-GlcNAc, UDP-N-acetyl-3-O-(3-hydroxytetradecanoyl)-o-d-glucosamine; UDP-2,3-diacylglyceramine-1-P or lipid X, N,O-bis[(R)-3-hydroxytetradecanoyl]-o-d-glucosamine-1-phosphate; tetramethyldisaccharide 1-phosphate, O-[2-amino-2-deoxy-\(N\)-\(O\)-bis[(R)-3-hydroxytetradecanoyl]-\(\beta\)-d-glucopyranosyl]-[1-6]-2-amino-2-deoxy-\(N\)-\(O\)-bis[(R)-3-hydroxytetradecanoyl]-α-D-glucopyranose-1-phosphate; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate.
SDS-Polyacrylamide Gel Electrophoresis—The discontinuous polyacrylamide gel system was basically that described by Laemmli (20). Gels of (0.75 mm x 6.5 cm x 8 cm (17.5% polyacrylamide) with 10 wells on the stacking gel were used for analyzing the purity of the enzyme. The samples were prepared by precipitation on ice with 10% trichloroacetic acid overnight, followed by washing with HPLC-grade acetone, resuspension, and boiling for 1.5 min in sample buffer (0.0625 M Tris, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 0.001% bromophenol blue, and 0.1 M mercaptoethanol).

Protein Sequencing and Composition—The sample for protein sequencing was prepared by further purification of the heparin-agarose peak fractions, using SDS-polyacrylamide gel electrophoresis, under the same conditions as described above, except that running gels of 1.5-mm thickness were used, prepared with 5 wells in the stacking gel. Two lanes were loaded with the purified enzyme sample (0.1 mg in 50 μl) and one lane with molecular weight standards. After electrophoresis, the portion of the gel containing the standards and one-fourth of one lane containing the sample were cut out and the proteins stained quickly (0.04% Coomassie Brilliant Blue G in 3.5% perchloric acid). The portion of the unstained gel corresponding to the stained lipid A disaccharide synthase band was cut out, crushed into small pieces, and left soaking in 0.5 ml of 0.05 M NH₄HCO₃ and 0.1% SDS at 07 C overnight. After centrifugation, the buffer was removed and the precipitate was washed once with 1.5 ml of 4.5% using formic acid solution. The protein thus eluted (~1 ml) was subjected to exchange into 0.01 M NH₄HCO₃ by five cycles of concentration and dilution using a Centricon YM30 (Amicon) ultrafiltration unit. The concentrated sample was lyophilized and submitted for protein sequencing.

The isolation of the temperature-sensitive E. coli strain MN7 from E. coli strain MN7 using a convenient new procedure for obtaining 0.1-1 g of pure material. All solvents and chemicals employed were of reagent grade. Methanol and pyridine were redistilled prior to use. Formic acid (88%) was used without redistillation. DEAE-cellulose (Whatman DE52 microgranular) for column chromatography was washed extensively with distilled water and equilibrated with 2.4 M ammonium acetate prior to use. Silicic acid for column chromatography (Bio-Sil A, 200–400 mesh) was obtained from Bio-Rad. Silica Gel 60 thin layer plates were obtained from E. Merck, Darmstadt, Federal Republic of Germany. Yeast extract and tryptone were obtained from Difco.

The isolation of the temperature-sensitive E. coli strain MN7 (ATCC no. 39528) has been described (17). The cells were grown in a modified LB broth, which contains 10 g of NaCl, 10 g of Tryptone, 5 g of yeast extract, 4 g of glucose, 2.33 g of KH₂PO₄, and 1 g of KH₂PO₄/liter. Maximum accumulation of lipid X (without much lipid Y) occurred in the early stationary phase of growth. The culture was harvested at 30-35 °C. This procedure was employed routinely in a 300-liter fermentor at the Biochemistry Department Pilot Plant, University of Wisconsin-Madison. Cells were grown exponentially in the above medium until the A₅₇₄ was 1.5. At this point an additional 1.50 g of glucose was added to the fermentor, and growth at 30 °C was continued until A₅₇₄ reached 3.0. At this point an additional 3000 g of glucose was added to the fermentor, and the temperature of the fermentor was shifted from 30 to 43.5 °C over a period of approximately 10 min. The pH was maintained at 7 throughout the fermentation by addition of concentrated ammonium hydroxide, and dissolved oxygen concentration was maintained at approximately 10% by aeration. At 43.5 °C, cells were harvested by centrifugation through a Sharples continuous flow centrifuge, and the cell paste was stored at 80 °C.

With the improved conditions described above, the yield of cell paste/300-liter fermentation was approximately 3 kg, about 4-5-fold greater than in our earlier work (4).

Frozen cells (600 g) prepared as described above were broken into chunks with a hammer and suspended in 1 liter of methanol and 500 ml of chloroform. The mixture was stirred occasionally and allowed to thaw for about 30 min. Next, the chunks were disrupted completely by a brief homogenization in a Waring blender, and the slurry was filtered in two equal portions to remove solid particles using two Buchner funnels (24 cm in diameter) equipped with 4-liter side arm vacuum flasks. The extracted lipids emerge as a clear yellow solution. The solid residue forms a gray cake on the funnel, which can be removed with a vacuum pump. At this stage, 498 mg of lipid X were recovered. The material was greater than 97% pure as judged by thin layer chromatography of 3-μl samples, followed by charring.

The lipid X emerged in fractions 4-8, but fraction 4 was discarded because it contained more rapidly migrating impurity. Fractions 5-8 were pooled, dried by rotary evaporation, and residual solvent was removed with a vacuum pump. At this stage, 498 mg of lipid X were recovered. The lipid X is extracted with 200 ml of chloroform/methanol (2:3:1, v/v) removing undesired pigments, phosphatidic acid and cardiolipin. Next, the column was washed with 200 ml of chloroform/methanol (9:1, v/v), and then the lipid X was eluted with 200 ml of chloroform/methanol (1:1, v/v). The final portion was collected, giving a total volume of 600 ml. Next, the solvent proportions were changed by adding 100 ml of chloroform, 160 ml of water, and 10 ml of concentrated HCl. After mixing in a separatory funnel, the phases were allowed to separate, and the lower phase was dried by rotary evaporation. Fractions at 10 ml per fraction were collected by thin layer chromatography of 3-μl samples, followed by charring. The lipid X emerged in fractions 4-8, but fraction 4 was discarded because it contained a more rapidly migrating impurity. Fractions 5-8 were pooled, dried by rotary evaporation, and residual solvent was removed with a vacuum pump. At this stage, 498 mg of lipid X were recovered. The material was greater than 97% pure as judged by thin layer chromatography and ¹H NMR spectroscopy in CDCl₃/CD₃OD (2:1, v/v). The spectrum was identical to that of a chemically synthesized standard provided by I. Macher and F. Unger of Sandoz (22) (data not shown).

To remove traces of lipid Y and other mitogenic impurities that cannot be detected by analytical methods other than bioassays, a portion of the lipid X was further purified by reverse phase column chromatography (Baker octadeylsilane, flash chromatography bulk packing, C₁₈ bonded to silica gel, 40-μm particles). Two solvent
mixtures (HPLC grade) were employed for this purpose: solvent A, acetonitrile, 5 mM t-butyrammonium phosphate in water (1:1, v/v); and solvent B, isopropyl alcohol, 5 mM t-butyrammonium phosphate in water (85:15, v/v). A 20-ml column (1.15 x 1.5 cm) of the reverse phase packing suspended in solvent A/solvent B (2:1, v/v) was poured and washed with 60 ml of the same mixture. Next, 200 mg of Bio-Sil A-purified lipid A disaccharide, dissolved in 20 ml of solvent A/B (2.1, v/v) and applied to the column at its natural flow rate. The column was washed with 60 ml of A/B (2.1, v/v), 60 ml of A/B (1:1, v/v), 60 ml of A/B (1:2, v/v), and 60 ml of B. Twelve fractions (20 ml each) were collected. Most of the lipid X emerged in fractions 4 and 5, which were combined.

The t-butyrammonium phosphate was removed by a final ion exchange chromatography. A 25-ml column of DE52, prepared as described above, was washed with 75 ml of chloroform/methanol/water (2:3:1, v/v), and the sample (fractions 4 and 5 from the reverse phase column) was applied directly. Next, the column was washed with 50 ml of chloroform/methanol/water (2:3:1, v/v), and then the lipid X was eluted with 140 ml of chloroform/methanol, 240 mM ammonium acetate in water (2:3:1, v/v). Fractions of 20 ml were collected, and most of the lipid X emerged in fractions 2-5. These fractions were pooled, and 13 ml of CHCl3, 23 ml of water, and 1.4 ml of n-propanol were added. After mixing in a vortex mixer and centrifuging in a microfuge, the lower phase was removed, neutralized with 2 ml of distilled pyridine, and concentrated under dry nitrogen. The residual solvent was removed using the fluorescence enhancement of disaccharide synthase.

Disaccharide Synthase Assay Conditions—The critical micelle concentration of lipid

RESULTS

Purification of Lipid A Disaccharide Synthase of E. coli—In previous studies we have described the construction of an L-arabinose-inducible hybrid plasmid (pSR8) that directs the 200-500-fold overproduction of the lipid A disaccharide synthase in strains of E. coli (13). As in wild-type cells (7), the overproduced synthase was recovered predominantly in the 150,000 × g sup. supernatant fraction, which was employed as the starting material for the purification (Table I).

A preliminary survey of various ion exchange resins revealed that most of the enzyme bound to DEAE-cellulose or DEAE-agarose, but not to the corresponding carboxymethyl resins. Elution from DEAE-cellulose occurred at 0.2-0.25 M NaCl but did not result in more than a 2-fold purification (data not shown). Binding to and recovery from DEAE-cellulose was improved by the presence of 0.05-0.2% Triton X-100, which was therefore included in all experiments. The enzyme was stabilized against thermal inactivation at 50°C by 20% glycerol (v/v), which was included as well.

A survey of several dye-ligand binding columns revealed that the synthase had a strong affinity for blue B and red A, providing the basis for the rapid purification scheme described under "Experimental Procedures." As shown in Table I and Fig. 1, lane 2, most proteins present in the membrane-free extract were not retained by a Matrex Gel Blue B, whereas the synthase was bound and could be recovered with a 59.5% yield when the column was washed with 2.5 M NaCl (Table I and Fig. 1, lane 3). A further 2-fold purification was achieved with a similar binding-elution protocol utilizing Reactive Red (Fig. 1, lanes 4 and 5). As a final step, the enzyme was chromatographed and concentrated on heparin-agarose, which also removed traces of pigments carried over from the dye columns.

The purity of the final preparation (Fig. 1, lane 7) was approximately 95%. The impurity at Mr ~ 28,000 appears to be UDP-GlcNAc acyltransferase, the first enzyme of the lipid

Estimates of the Critical Micelle Concentration of Lipid X under the Disaccharide Synthase Assay Conditions—The critical micelle concentrations of lipid X and UDP-2,3-diacylglycosamine were estimated using the fluorescence enhancement of 8-anilino-1-naphthalenesulfonic acid (Eastman, magnesium salt) as the probe (23). A 10 μM stock solution of 8-anilino-1-naphthalenesulfonic acid in 10 mM sodium-Hepes buffer, pH 8 (Solution A) was prepared. Base-line fluorescence at 25°C was measured at 480 nm with the excitation wavelength set at 350 nm, using a 1-cm cuvette in a JY3 Jobin-Yvon spectrofluorometer. Next, a 5 mM stock solution of lipid X was prepared in 10 mM Hepes, pH 8, also containing 10 μM 8-anilino-1-naphthalenesulfonic acid, and the mixture was subjected to a 2-min sonic irradiation in a bath sonicator (Solution B). Increasing amounts of Solution B were then added to Solution A, and the fluorescence at 480 nm was measured as the lipid X concentration was varied from 10 to 1 mg/ml. The critical micelle concentrations of UDP-2,3-diacylglycosamine and sodium dodecyl sulfate were estimated in an analogous manner, except that the SDS concentration was varied from 0.05 to 12 mM.

Gel Filtration of Lipid X and UDP-2,3-diacylglycosamine—[^2P] Lipid X (10^6 cpm) in CHCl3, prepared as described above, was dried with a stream of N2, and 2.5 mg of lipid X in 500 μl of 10 mM Hepes, pH 8, was added. The mixture was subjected to a 2-min sonic irradiation in a bath sonicator, unless otherwise indicated. A portion of the material (440 μl) was applied to a 44-ml column of Sepharose CL-4B-200 (25 x 1.5 cm) equilibrated at 25°C with 10 mM Hepes, pH 8, and previously calibrated with blue dextran and [3H]inositol. The flow rate (0.23 ml/min) was controlled with a pump, and 1.3-ml fractions were collected.[^2P]Lipid X was detected by liquid scintillation counting. In some experiments, [3H]inositol (6 x 10^5 cpm) was added to the same to provide an estimate of the included volume. In experiments to estimate the size of UDP-2,3-diacylglycosamine micelles, the lipid was detected by measuring A262.

TABLE I

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<td>4. Heparin-agarose</td>
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The lpxB Gene Product

A pathway (data not shown). The observed subunit molecular weight of the synthase ($M_r \sim 42,000$) is in agreement with previous minicell radiolabeling experiments and DNA sequencing (13, 14). The final heparin-agarose fraction (Table I) is purified 54-fold relative to the membrane-free supernatant, but since the latter has a specific activity that is $\sim$300-fold above wild type, the nearly homogeneous synthase is enriched $\sim$16,000-fold relative to wild-type extracts. This purification factor is comparable to that observed for enzymes involved in glycerophospholipid biosynthesis in E. coli (3, 24).

Amino-terminal Sequence and Amino Acid Composition of the Purified Lipid A Disaccharide Synthase—The amino-terminal sequence was obtained by sequential Edman degradation of a hybrid lpxB-lacZ fusion protein (14). The amino acid composition, determined directly on the lipid A disaccharide synthase at various stages of the purification. Lane 1, crude supernatant, 11 nmol/min; lane 2, equal volume of Matrex Gel Blue B run-through; lane 3, after elution from Matrex Gel Blue B, 7.5 nmol/min; lane 4, 10 $\times$ volume of Reactive Red-agarose run-through; lane 5, after elution from Reactive Red-agarose, 15.6 nmol/min; lane 6, 10 $\times$ volume of heparin-agarose run-through; lane 7, after elution from heparin-agarose, 10.0 nmol/min.

The lpxB Gene Product

The lpxB Gene Product

Amino acid composition of lipid A disaccharide synthase

The gene sequence has been reported previously (14). The theoretical monomer mass is 42,339 daltons.

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Identification of the Products Generated by the Disaccharide Synthase—To confirm that the purified enzyme generates the same tetraacyldisaccharide 1-P product previously synthesized in crude extracts and analyzed in detail by fast atom bombardment mass spectrometry and $^1$H NMR (7), a large-scale enzymatic reaction mixture (2 ml) was prepared containing 1 mM UDP-2,3-diacylglycerolamine, 1 mM lipid X, 5 presence of 0.05% Triton X-100, however, we cannot exclude the possibility that the enzyme is a monomer but is associated with detergent micelle(s). The latter possibility seems unlikely, however, given the fact that added Triton X-100 is not required for catalytic activity (see below). The proteins used to calibrate the column (Fig. 2) are not affected by the presence of Triton X-100.

Fig. 2. Gel filtration of the disaccharide synthase on Sephadex G-200. The enzyme sample (0.25 mg in 0.5 ml of heparin-agarose elution buffer) was applied to a Sephadex G-200 column (1.4 cm) equilibrated with 0.01 M potassium phosphate, pH 7.0, 0.2 M KCl, 20% glycerol, and 0.05% Triton X-100. The standards used to calibrate the column were not influenced by the presence of Triton X-100.
possible to identify UDP as a product of the disaccharide synthase reaction because of further metabolism of this nucleotide in crude extracts, primarily to UMP and uracil. With the purified enzyme, however, it was possible to demonstrate enzyme and time-dependent formation of UDP (data not shown), using a reverse phase HPLC system (27). In addition, the 31P NMR spectrum at 81 MHz of the dried upper Bligh-Dyer phase (see above), redissolved in 0.5 ml of D2O at 25 °C, was identical to that of an authentic UDP standard (Fig. 3) and was typical of an asymmetrically substituted pyrophosphate.

Nucleotide and Fatty Acyl Specificity of the Lipid A Disaccharide Synthase—As shown in Table III, the uridine moiety was strongly preferred by the enzyme, but significant product formation was observed with all the substrates shown. In living cells, the participation of bases other than uracil, or possibly thymine, is very unlikely, given the results of Table III. Nevertheless, when a large amount (100-1000 μg/ml) of the pure disaccharide synthase is employed, efficient utilization of UDP-3-O-((R)-3-hydroxymyristoyl)-GlcNAc for disaccharide formation is possible in vitro (data not shown).

Using the qualitative thin layer assay (see "Experimental Procedures"), we have also examined the specificity of the disaccharide synthase for the reducing end moiety. Mild alkaline hydrolysis of lipid X yields N-(3)-dihydroxymyristoyl)-GlcN-1-P, which is utilized by the disaccharide synthase at ~0.01% of the rate of 1 mM lipid X (data not shown). Chemically synthesized 2,3-diacylglycerol-GlcN-1-P (prepared by using the methods described in Ref. 22) is also utilized as a substrate at 1 mM, but again, only at 0.01% of the rate of 1 mM lipid X (data not shown). Lipid Y and its UDP derivative are not substrates under our conditions. With the highly purified synthase, we have not detected the hydrolysis of UDP-2,3-diacylglycerol, even after a 24-h incubation in the presence of 1 mg/ml protein (data not shown).

The ability of the enzyme to accept certain analogs of 2,3-diacylglycerol depends on the rate, at a slow rate, is very useful for the
rapid semisynthetic preparation of numerous closely related lipid A analogs and substructures (15).

**Kinetic Properties of the Lipid A Disaccharide Synthase—**
Despite the hydrophobic nature of the substrates employed, the disaccharide synthase displays apparently simple saturation kinetics over a wide range of substrate concentrations examined (Fig. 4). In panel A, the UDP-2,3-diacylglycosamine concentration was set at 1 mM, and lipid X was varied as indicated. When fit by computer (28) to a two-substrate Michaelis-Menten program, values of \( V_{\text{max}} = 14,028 \pm 513 \text{ nmol/min/mg} \) and \( K_m = 0.266 \pm 0.023 \text{ mM} \) were obtained for the apparent kinetic constants. Conversely, when the concentration of lipid X was maintained at 1 mM and the UDP-2,3-diacylglycosamine was varied (panel B), \( V_{\text{max}} \) was calculated to be \( 12,370 \pm 472 \text{ nmol/min/mg} \) and \( K_m = 0.114 \pm 0.014 \text{ mM} \). The significance of these apparent constants must be considered in light of the fact that both substrates are present as aggregates at concentrations greater than 20 \( \mu \text{M} \) (see below). The relatively high apparent \( K_m \) values support the view that the synthase acts on aggregates of lipid X and UDP-2,3-diacylglycosamine, rather than on monomers.

An additional unusual feature of the synthase and its substrates is revealed by the results of Fig. 5. There is no detectable activation of the reaction by added Triton X-100. This behavior contrasts with that of all known phospholipid synthetic enzymes of *E. coli* that utilize diacylglycerolphospholipid substrates (3, 24), since these enzymes have an absolute requirement for the addition of nonionic detergent under in vitro assay conditions, when purified to homogeneity. At high concentrations of Triton (above 1%, Fig. 5) slight inhibition of the disaccharide synthase is observed, perhaps reflecting surface dilution of both substrates in mixed Triton X-100/substrate micelles that would be expected to form under those conditions (29, 30).

**Physical State of Lipid X and UDP-2,3-diacylglycosamine under the Conditions Used to Assay the Lipid A Disaccharide Synthase**—The apparent simplicity of the steady state kinetics (Fig. 4) and the lack of dependence of the enzymatic activity on the presence of a detergent (Fig. 5) suggested that lipid X and UDP-2,3-diacylglycosamine might have physical properties distinct from those of the common glycerophospholipids (31). Accordingly, we examined the aggregation state of lipid X and UDP-2,3-diacylglycosamine prepared as dispersions in water at pH 8. As shown in Fig. 6, the critical micelle concentration of both compounds is less than 20 \( \mu \text{M} \), as judged by measurement of the enhancement of 8-anilino-1-naphthalenesulfonic acid fluorescence. (Control experiments with sodium dodecyl sulfate gave the expected critical micelle concentration of \( 4.5 \text{ mM} \) (31).) The finding that the critical micelle concentrations of lipid X and UDP-2,3-diacylglycosamine are below 20 \( \mu \text{M} \) strongly argues that the disaccharide synthase acts on substrate aggregates, rather than monomers, given the saturation curves shown in Fig. 5. The actual critical micelle concentrations can be expected to be in the range of \( 10^{-8} \text{ M} \), assuming that analogies with phosphatidylcholine are valid (31). The exact values will have to be determined directly by more sensitive radiochemical methods (32).

To estimate the size of the lipid aggregates, gel filtration experiments were performed at pH 8 using Sepharose CL-4B (Fig. 7). The excluded volume was determined with blue dextran, and the included volume was estimated with \( [3\text{H}] \) inositol (Fig. 7, panel A). If lipid X (prepared as a 5 mM stock in 20 mM Hepes, pH 8) was chromatographed without sonic

![Fig. 4. Kinetic properties of the purified disaccharide synthase. Panel A, the concentration of UDP-2,3-diacylglycosamine was held at 1 mM, and the concentration of lipid X was varied. The apparent \( V_{\text{max}} \) and \( K_m \) are \( 14,028 \pm 513 \text{ nmol/min/mg} \) and \( 0.266 \pm 0.023 \text{ mM} \), respectively. Panel B, the concentration of lipid X was held at 1 mM, and the concentration of UDP-2,3-diacylglycosamine was varied. In this case the apparent \( V_{\text{max}} \) is \( 12,370 \pm 472 \text{ nmol/min/mg} \) and the apparent \( K_m \) is \( 0.114 \pm 0.014 \text{ mM} \). The lines were generated by fitting the data to the Michaelis-Menten equation using a previously described computer program (28). The actual concentration of lipid X and UDP-2,3-diacylglycosamine micelles is several hundred-folds lower than the indicated chemical concentrations of the lipids.](image)

![Fig. 5. The effect of Triton X-100 on the activity of the lipid A disaccharide synthase. Both lipid X and UDP-2,3-diacylglycosamine were held at 1 mM each, and the concentration of Triton X-100 was varied, as indicated.](image)
The lipX Gene Product

**Fig. 6.** Determination of the critical micelle concentrations of lipid X, UDP-2,3-diacylglucosamine, and sodium dodecyl sulfate by their ability to enhance the fluorescence of 8-anilino-1-naphthalenesulfonic acid. Within experimental error, there was no evidence for a plateau region in the 0–0.2 mM concentration range, either for lipid X or for UDP-2,3-diacylglucosamine. From this data, we can estimate the critical micelle concentrations of these substances to be less than 20 μM.

The lipX gene product is a peripheral membrane protein, not a true integral membrane protein. The previously published DNA sequence of the synthase (14) is consistent with this view, since no prominent hydrophobic clusters of amino acids capable of spanning the membrane are present in the synthase. We have not yet examined our preparations for the presence of other cofactors.

**Fig. 7.** Gel filtration of lipid X and UDP-2,3-diacylglucosamine on Sepharose CL-4B. Lipid samples were prepared at 5 mM (see "Experimental Procedures"). Brief sonic irradiation caused them to run as a discrete small peak, around the molecular weight of catalase (~240,000). There is no evidence for dissociation during chromatography to lower molecular weight species, consistent with the view that the critical micelle concentration is many orders of magnitude less than the chemical concentration. BSA, bovine serum albumin.

**Discussion**

The lipid A disaccharide synthase of *E. coli* has been purified to ~95% homogeneity, utilizing a strain that overproduces the enzyme ~300-fold relative to wild-type cells. This is the first example of the purification of an enzyme involved in lipid A biosynthesis in any system. Interestingly, the synthase is not membrane-bound, in contrast to most enzymes involved in glycerophospholipid biogenesis (3). Given the fact that at least one of its substrates (lipid X) is membrane-bound (35), however, it is likely that it carries out its catalytic function on the inner surface of the inner *E. coli* membrane in the sense of a true peripheral membrane protein. The previously published DNA sequence of the synthase (14) is consistent with this view, since no prominent hydrophobic clusters of amino acids capable of spanning the membrane are present in the synthase. We have not yet examined our preparations for the presence of other cofactors.

The 42,000-dalton protein observed in the purified preparations by SDS gel electrophoresis (Fig. 1) can be identified as the lipid A disaccharide synthase for the following reasons.

1) The molecular weight, N-terminal sequence, and amino acid composition of the purified protein match the predicted protein product deduced from the DNA sequences (14).
2) The extensive purification required relative to wild-type extracts of *E. coli* (i.e. ~16,000-fold) is
comparable to that required for constitutive enzymes involved in glycerophospholipid synthesis, such as CDP-diglyceride synthase (3, 24). We estimate that there are approximately $10^9$ disaccharide synthase monomers/wild-type cell. This value is somewhat less than that of lipid X ($10^9$ molecules/wild-type cell) but is comparable to that of UDP-2,3-diacylglycerolamine ($10^9$ molecules/wild-type cell) (6), suggesting that the disaccharide synthase is probably limited by the availability of substrate under ordinary conditions. In this regard, we have found that disaccharide synthase overproducing cells have normal levels of mature lipid A in their envelopes.

The unusual kinetic properties of the disaccharide synthase (i.e. the lack of a requirement for a nonionic detergent, Fig. 5) led us to examine, for the first time, the aggregation state of the glucosamine-derived phospholipids. As shown in Fig. 7, the observed aggregates are much smaller than predicted for liposomes or multilamellar vesicles [33] in which the component phospholipids are arranged in bilayers. This result was unexpected, given the presence of two relatively long hydrocarbon chains in each molecule of lipid X. Classical glycerophospholipids, having two hydrocarbon chains of 12 or more carbons in length, typically form bilayers when dispersed in water (31, 34). The micellar configuration of pure lipid X in water at pH 8 may be due to the relatively large size of the glucosamine 1-phosphate headgroup, its negative charge and extent of hydration, or possibly, the presence of the 0-hydroxy functions. The tendency of pure lipid X to form micelles does not preclude its insertion into bilayers when a large excess of glycerophospholipid is present, as would be the case during lipid A biosynthesis on the inner surface of the inner membrane.

In any case, a detailed analysis of the physical properties of the glucosamine-derived phospholipids, including lipid A and all its precursors, will be necessary in order to gain a full understanding of the interaction of these substances with enzymes and with the surfaces of animal cells, where they trigger a variety of complex pathophysiological effects (1, 2, 5).

The difficulties encountered in the chemical synthesis of lipid A (36) have limited the preparation of probes that would be useful for the investigation of lipid A-animal cell interactions. For instance, fluorescent lipid A molecules would be useful for studying lipid A uptake and intracellular translocation (37), whereas photoactivatable lipid A analogs (38) would be helpful in defining receptors or active sites on proteins that recognize lipid A. We believe that the purified disaccharide synthase will be a very useful reagent with which to prepare interesting lipid A analogs, since preliminary studies indicate that the specificity of the enzyme for fatty acyl chain length and carbohydrate backbone is not absolute (15). The fact that the enzyme has a strong kinetic preference for long chain diacyl analogs (Table III), together with its high apparent $K_a$ values relative to the critical micelle concentration of its substrates, suggests that it may function optimally on aggregated lipid surfaces, perhaps like the soluble phospholipase A$_2$ from pancreatic juice or bee venom (30).

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REFERENCES